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14. ABSTRACT

This project is an investigation of the involvement of the enzyme arginase type II (AII) in the pathogenesis and growth of prostate cancer. Having cloned the AII gene in our laboratory, we unexpectedly discovered that is expressed at high levels in the normal prostate and even higher in neoplastic prostate samples. The purpose of the present research funded by USAMRMC is to examine the expression of AII in a wider range of benign and malignant prostate specimens and cultured cells to determine its usefulness as a novel marker of prostatic neoplasia and the extent of its involvement in cancer pathogenesis. We are also exploring whether specific chemical and molecular inhibitors of arginase and several related enzymes in the polyamine metabolic pathway might suppress or arrest the growth of prostate cancer cells in vitro or in vivo. This third annual report describes our progress over the past year in extending our characterization of arginase and other related enzymes in prostate cancer cell lines of various degrees of differentiation using more quantitative analysis, correlating polyamine synthesis in these cell lines, creating siRNA constructs that can be stably expressed in these lines, expanding our prostate tissue studies to include tissue microarrays, and assessing prostate tumors for the proposed in vivo studies.

15. SUBJECT TERMS
Prostate cancer, arginase, polyamines

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Introduction

Prostate cancer is the most common and second most lethal cancer among men in the United States, yet despite its high prevalence, relatively little is known about the biochemical and molecular mechanisms controlling benign and malignant prostatic growth. We have proposed to investigate the involvement of the enzyme arginase type II (AII) in this malignancy. We believe that this enzyme plays a pivotal role in the synthesis of polyamines, chemicals involved in cell growth and regulation that are found in high levels in normal prostate tissue and in cancer cells. Having cloned the AII gene in our laboratory, we unexpectedly discovered that it is expressed at high levels in the normal prostate and even higher in neoplastic prostate samples. The purpose of the present research funded by USAMRMC is to examine the expression of AII in a wider range of benign and malignant prostate specimens and cultured cells to determine its usefulness as a novel marker of prostatic neoplasia and the extent of its involvement in cancer pathogenesis. We also are exploring whether specific chemical and molecular inhibitors of arginase and several related enzymes in the polyamine metabolic pathway might suppress or arrest the growth of prostate cancer cells *in vitro* or *in vivo*. The specific aims of the project are to determine: (1) the specific prostate cell types responsible for the high-level expression of arginase AII, (2) the role of arginase in critical pathways of polyamine and nitric oxide synthesis in benign and malignant prostatic growth, (3) the mechanism and efficacy of targeted molecular and biochemical inhibitors of the arginase pathway in blocking the growth of prostate cancer cells, (4) the effect of genetically engineered overexpression of arginase and related enzymes on prostate cancer cell growth, and (5) the significance of arginase AII activity as a potential novel diagnostic marker and/or therapeutic target of prostatic neoplasia *in vivo*. We believe this work will shed new light on the fundamental mechanisms of prostatic neoplasia while at the same time suggesting new directions for diagnosis and therapeutic intervention.

Body of Report

Following a longstanding interest in the first discovered liver isoform of arginase (AI) as the focus of a rare inborn error of metabolism (arginase deficiency; hyperargininemia), our laboratory more recently began to focus on the role of the second, extrahepatic isoform, arginase II (AII) and its potential as a novel marker for prostate cancer. Arginase II is highly expressed in the normal prostate and even more highly expressed in patients with prostate cancer. A major overarching goal of the funded project is to artificially engineer cells, tissues, and model organisms (mice) to achieve overexpression or inhibition of the arginase isozymes and other related genes and enzymes of arginine metabolism and study the effect on prostate cancer cell growth. Much of the initial year of the project was spent developing and testing the necessary reagents for manipulating these genes in the various target milieus specified in the grant proposal. The second year saw the application of these reagents to the *in vitro* experiments proposed and to a variety of benign and malignant human prostate tissue samples. The third year has focused on expanding the initial cell culture and prostate tissue experiments while at the same time extending our studies to include the proposed *in vivo* models.

The key genes we have chosen to focus on, by virtue of our postulated involvement of them in prostate cancer growth, are arginases I and II, ornithine decarboxylase (ODC), agmatinase (Agm), ornithine aminotransferase (OAT), and arginine decarboxylase (ADC). Each of these enzymes functions within the extended arginine metabolic pathway (Fig. 1), which our group, primarily in a companion grant from the NIH headed by Dr. Stephen Cederbaum, has been working out in the context of a rare inborn error of metabolism, arginase deficiency (hyperargininemia).

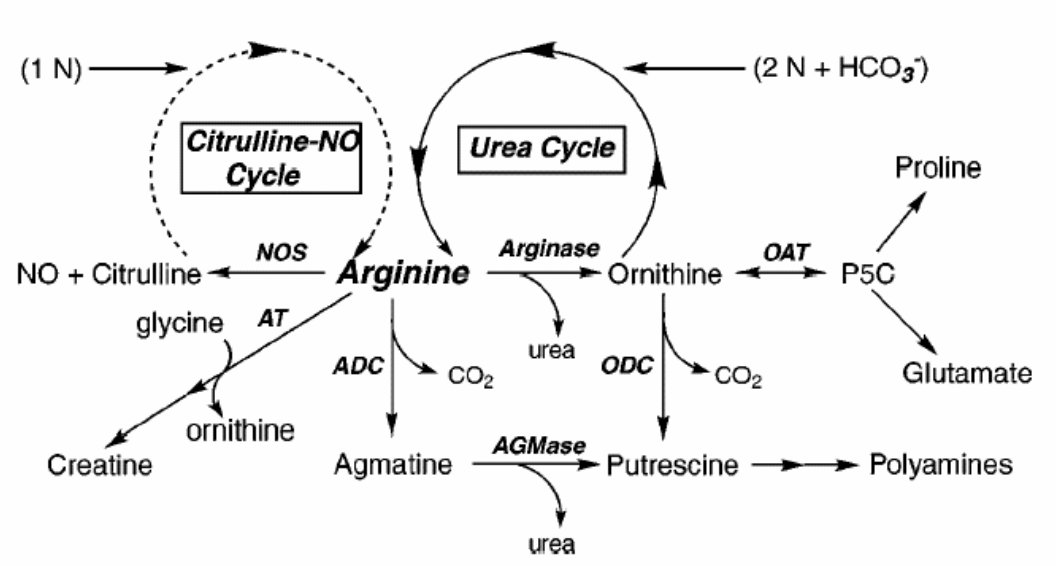

Figure 1. The Arginine Metabolic Pathway, Showing Side Reactions toward Polyamine Synthesis (Morris, 2002).

During the first year we constructed overexpression elements containing GFP (green fluorescent protein) fusions under the control of the CMV constitutive promoter for each of the above genes, and have tested them using a variety of enzymatic assays. In the second year we used these elements to create stable overexpression prostatic tumor cell lines. In addition, interfering RNA (siRNA) constructs (Lieberman et al, 2003) were further optimized using RT-PCR and western blot analysis. We also began to study effects on polyamine synthesis, which according to our hypothesis is the focal point of arginase effect on tumor cell proliferation. Much of the third year was spent gathering more information on the family of genes involved in the arginine and polyamine pathways through quantitative real-time PCR and polyamine studies. We have also started our proposed *in vivo* studies, which should mirror the results we have achieved from both the cell culture and tissue experiments conducted thus far.

A summary of these accomplishments follows, with reference made to the specific items in the approved Statement of Work to which they apply.

Task 1.a Arginase Isoforms in Prostatic Tissue and Cell Lines

Most of our cell culture experiments thus far have encompassed the well-characterized prostatic cancer cell lines, LNCaP, PC3, and DU145. Recently, we expanded our studies to include two new prostate cell lines, LAPC-4 and TRAMP-C2. The LAPC-4 line is a unique androgen-sensitive human prostate cancer cell line that we acquired through our colleague within the UCLA Cancer Center, Dr. C. Sawyers (Klein *et al.* 1997). Dr. N. Greenberg developed the TRAMP-C2 cell line from a primary prostate tumor of a 32-week-old TRAMP mouse (Foster *et al.* 1997). As mentioned in our last report, the TRAMP (transgenic adenocarcinoma mouse prostate) transgenic mouse line carries the SV40 large T and small t tumor antigens under an androgen-driven probasin promoter that is designed to develop prostate cancer. We used western blot analysis to determine the amount of AII protein in the LAPC-4 and TRAMP-C2 cell lines along with the expression levels observed in LNCaP, PC3, and DU145 (Fig. 2). All of the above mentioned tumor cell lines can be compared to AII expression seen in PZ-HPV-7, an immortalized cell line representing essentially normal prostate. The preponderance of our data thus far show that AII activity is most prominent in the more differentiated prostate cancer cell lines (LNCaP, LAPC-4), with lower expression observed in the normal prostate (PZ-HPV-7) and in the more undifferentiated cancer cells (PC3, DU145). AII expression appears to be absent from the TRAMP-C2 cell line which is consistent with our hypothesis that as tumors become more undifferentiated and representative of late-stage cancer, AII activity seems to diminish.

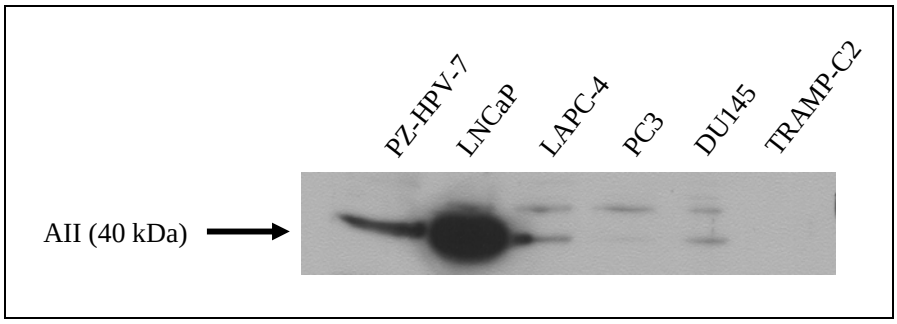

Figure 2. AII protein analysis in PZ-HPV-7, LNCaP, LAPC-4, PC3, DU145, and TRAMP-C2 prostate cell lines by western blot.

We believe that arginine and its immediate metabolic products lie at the center of an important biochemical nexus, acted upon by a number of complementary and competing enzymes, including arginases AI and AII, OAT, ODC, Agm, and ADC. In a previous report, we analyzed LNCaP, PC3 and DU145 cells using semiquantitative RT-PCR for all of the above mentioned genes. Since then, our lab has adopted the technique of real-time PCR, a quantitative assay that provides a more precise understanding of the expression levels of these related genes in prostate cell lines. In addition to looking at these genes in LNCaP, PC3 and DU145, we expanded our preliminary study to include both LAPC-4 and TRAMP-C2 cell lines (Fig. 3). The expression levels observed in these prostate cancer cell lines is relative to the expression seen in PZ-HPV-7, which was given an arbitrary value of 1. Each cell line fell above or below the control PZ-HPV-7, dependent upon the expression level of the gene being evaluated. It appears that expression of particular genes, such as AII and ODC are elevated in the more differentiated cell lines (LNCaP, LAPC-4) and are low in the more undifferentiated cell lines (PC3, DU145). The opposite trend is observed with OAT and ADC, which are greater in the more undifferentiated cell lines.

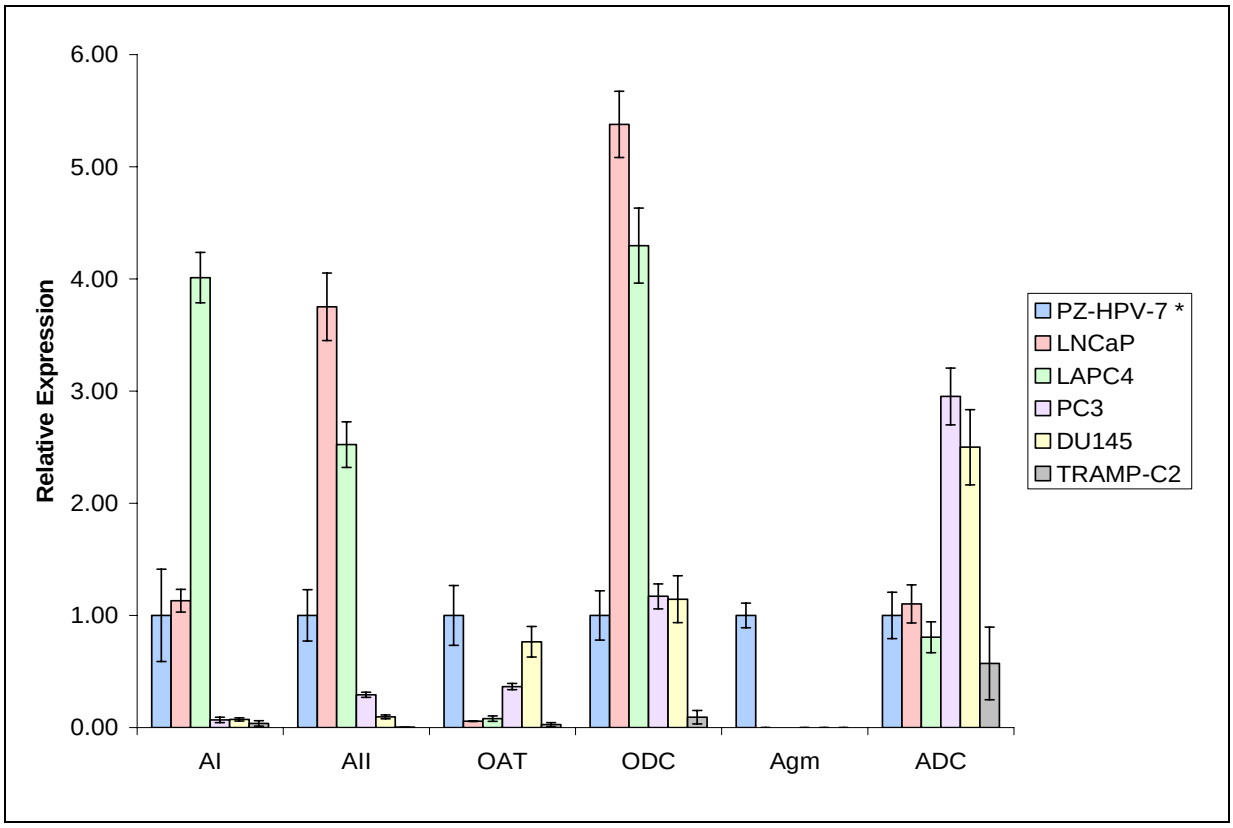

Figure 3. Quantitative Real-Time PCR analysis for expression of selected arginine and polyamine metabolic genes in PZ-HPV-7, LNCaP, LAPC4, PC3, DU145, and TRAMP-C2 prostate cell lines.

Task 1.b In Situ Hybridization and Immunohistochemistry

In our last report, we had begun looking at AII expression in frozen human prostate tissue samples that we obtained from the Tissue Procurement Core Facility at UCLA. We have now extended this study to a greater number of specimens, including paraffin-embedded tissues. We performed immunohistochemical analysis on various tissues from primary prostatectomry cases stained with a specific AII antibody that we have used in our previous work (Fig. 4). We found that paraffin sections offered better staining definition and tissue quality compared to the frozen sections. We noticed that with the paraffin sections, AII activity was solely localized to the glandular epithelial cells and not observed in the stroma of the prostate. A similar staining pattern was noted among the various tissue types in both frozen and paraffin sections. A gradient in glandular AII expression was observed where normal tissue expressed the least AII with the strongest signal seen in malignant tissue. In addition to this observation, we saw significantly stronger AII expression in the lower Gleason grade tissues with a decline in AII expression as tumors become more undifferentiated and characteristic of late-stage cancer. This is consistent with our hypothesis that more differentiated tumors express higher levels of AII, but as the tumors progress and become more undifferentiated AII expression seems to diminish. These findings, in conjunction with the *in vitro* data mentioned earlier, lend further strength to the notion that AII might be playing a role in the development and progression of prostate cancer.

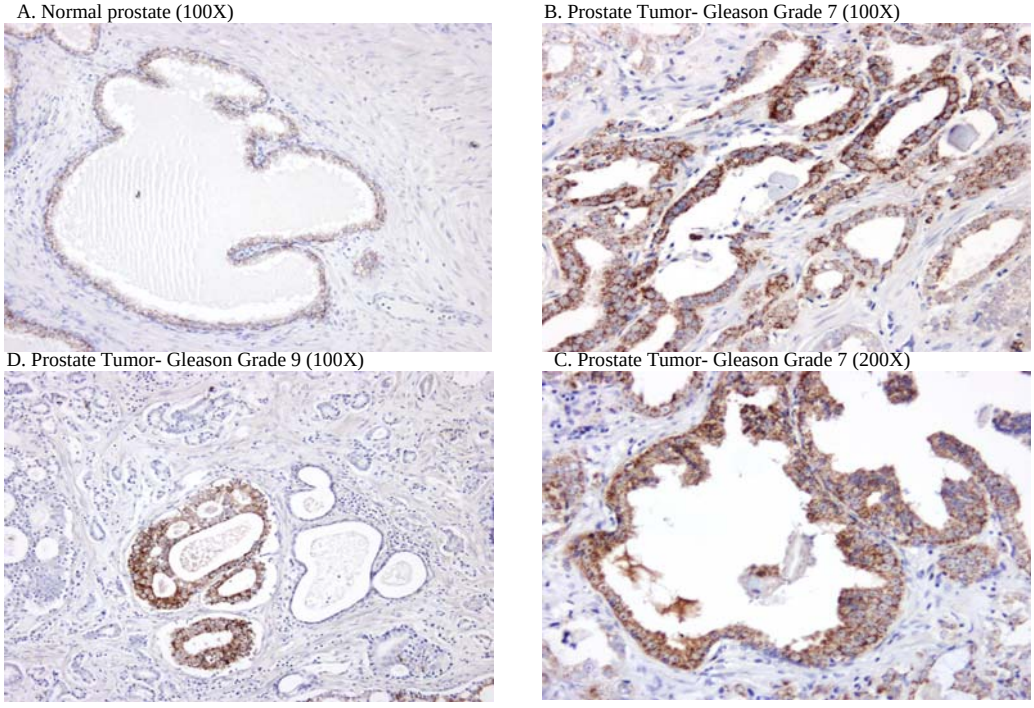

Figure 4. Immunohistochemical detection of arginase II expression in the glandular epithelial region of human prostate tissue samples.

To confirm this finding, we set-up a collaboration with Dr. D. Seligson in the UCLA Tissue Microarray Core, where we obtained a prostate tissue microarray with 1002 different tissue targets containing normal, benign prostatic hyperplasia (BPH), and all Gleason grades of prostate malignancy. Fig. 5 is a sample tissue microarray shown at different

magnifications stained with hematoxylin and eosin (H&E) to view morphology. We performed immunohistochemistry analysis for AII expression on the various prostate tissue targets and the data thus far (analyzed extensively by multivariate statistical methods) are not invariant. Several cases followed our particular hypothesis showing greater AII staining in the tumor versus the normal tissue target. There were even examples where within an individual tissue punch, the normal cells stained much less than the cancerous cells (Fig. 6). However, the variable AII staining intensity between tissue sections and even between neighboring prostate glands within the same tissue suggests a more complex interaction between arginase and other enzymes in the arginine metabolic pathway.

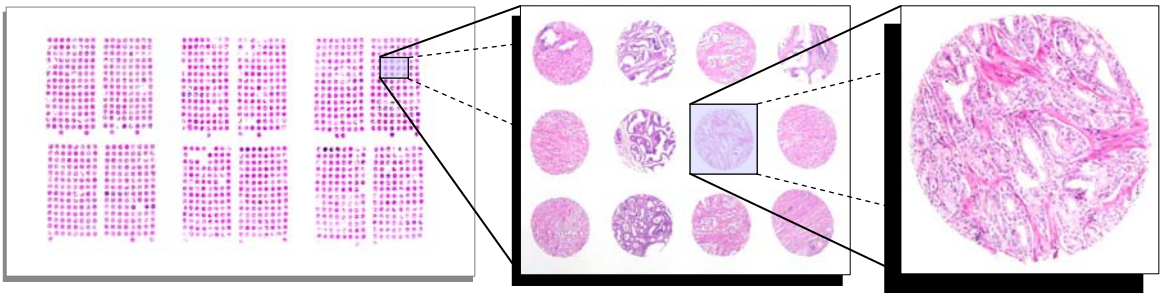

Figure 5. Sample tissue microarray at various magnifications; H&E stained tissue.

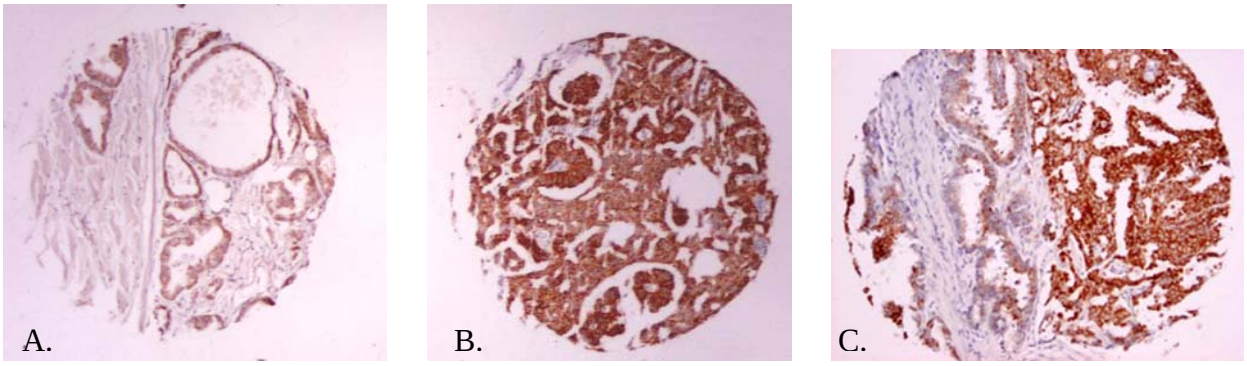

Figure 6. Tissue microarray spots stained with AII; (A) normal prostate with low levels of AII staining, (B) malignant prostate with high AII expression, (C) both normal and cancerous cells within individual spot; less AII expression in normal versus malignant area of tissue.

To further understand this interrelationship, we are now conducting real-time RT-PCR analysis on specific glandular regions microdissected using the laser-capture microscope (Arcturus, Mountain View, CA) available in the UCLA Tissue Procurement Core Laboratory. Our approach consists of dissecting out individual glands expressing high levels of AII and glands expressing little to no AII and comparing expression levels of the various genes involved in arginine metabolism, polyamine synthesis and genes found to be altered in the tumor microenvironment (HIF-1α, NADPH oxidase). There is a great deal of optimization that goes into performing this experiment, which is now in progress. In order to perform gene expression analysis on the individual glands being dissected, we must be able to extract intact RNA from the target tissue and amplify it to achieve a sufficient concentration for real-time PCR experiments. To insure that the RNA from our frozen prostate tissue samples was still intact and that we could achieve an ample amount for future experiments, we performed a tissue scrape protocol and two

rounds of RNA amplification (protocols and reagents by Arcturus). We were able to extract and amplify enough RNA from the sections and we are confident that the same results will be achieved when performing the actual experimental samples. Since our experiment relies on the ability to discern between glands staining with or without AII, we must perform a quick immunohistochemistry procedure on the frozen prostatic tissue sections prior to using the laser capture microscope. This will allow us to visually identify which glands are expressing AII. We are near completing the optimization process for this study based on recently published procedure (Rupp *et al.* 2006) and are about to begin the actual laser-capture microscope experiments. This study is aimed at uncovering the interaction among a multitude of related genes in an individual cell. Our hope is that we will eliminate the variability between whole tissue tumor sections, which are quite heterogeneous, by separating out cells expressing or not expressing AII.

Furthermore, to comprehensively study these interactions we will look for significant alterations in genes involved in transformation and tumorigenesis with the Human Cancer Pathway Finder PCR Array (SuperArray, Frederick, MD). Using real-time PCR, we will analyze the expression of genes implicated in cell cycle control, apoptosis, adhesion, angiogenesis, invasion and metastasis (i.e. p53, Bcl-X, NFKβ, Myc, MMP). These studies may allude to a biological pathway that is being modified by the presence or absence of AII.

We have decided to set aside the *in situ* hybridization experiments for now, since we are embarking on the laser-capture microscope studies that should provide us with similar results. The laser-capture microscope analysis will be using RNA to help define gene expression differences and will generate additional information on a wide variety of genes.

Task 2.a Polyamine Measurements

From the previous report, we have expanded our preliminary polyamine analysis to include PZ-HPV-7, LNCaP, PC3, and DU145 prostate cell lines (Fig. 7). When comparing polyamine levels in these cell lines to AII expression shown previously in Fig. 2, there appears to be a correlation between AII activity and polyamine production. LNCaP cells, which have high AII expression, have increased spermine levels and decreased spermidine levels. In contrast, PC3 cells have low levels of AII expression and an opposite trend for polyamine production. This suggests that AII levels may be contributing to the production of polyamines and in turn altering cell growth and proliferation. We are in the process of extending our polyamine studies to include the LAPC-4 and TRAMP-C2 cell lines to better understand the complex relationship between the arginine and polyamine pathways. The effect of AII inhibition on polyamine production *in vivo* is also an important aspect of this study. We have sent away to our collaborating testing laboratory the first set of prostates from male AII knockout and wild type mice and are awaiting complete polyamine analysis by HPLC. We suspect that polyamine levels will be diminished in the AII knockout prostates compared to wild type unless genes involved in an alternate pathway (i.e. OAT) are upregulated and compensate for the depletion of AII.

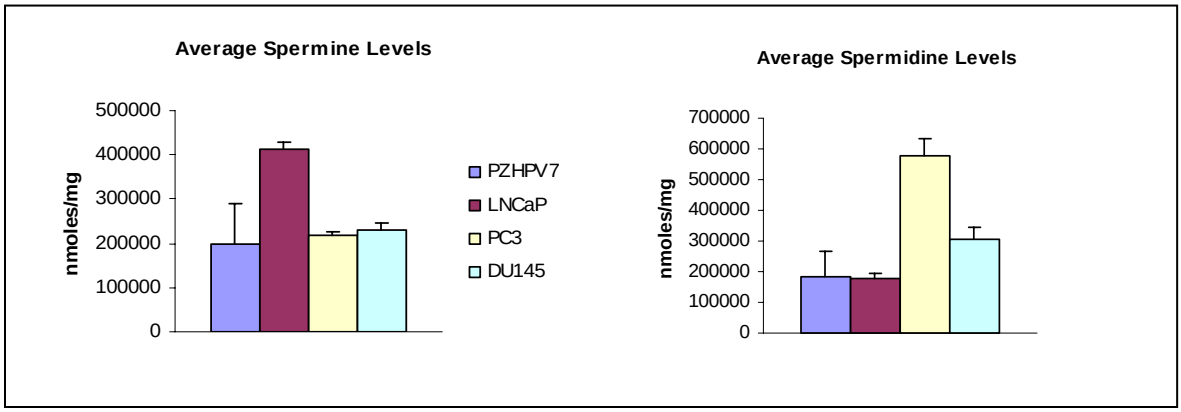

Figure 7. Polyamine levels for PZ-HPV-7, LNCaP, PC3, and DU145 cell lines

Task 2.b Polyamine Synthetic Enzyme Analysis

Expression levels for genes involved in the polyamine metabolic pathway were shown above in Fig. 3. In the near future, we will be performing a comprehensive comparison of the current real-time PCR data with the polyamine measurements from all of the prostate cell lines.

Task 2.c cDNA Miniarrays

As mentioned in our last report, we have decided to use microarray analysis rather than construct cDNA miniarrays to study variations in gene expression. We are in the process of optimizing the microarray technique for our cancer studies and during the interim we obtained real-time PCR primers for a number of genes related to tumor microenvironment (i.e. HIF-1α, NADPH oxidase). These results should preview our future microarray data and show how alterations in AII influence other biological pathways.

Task 3.a Inhibitors of Gene Expression

After determining the siRNA oligonucleotide with the highest AII knockdown percentage, we cloned it into a vector containing a neomycin selectable marker. We transfected LNCaP cells with this siRNA AII expressing vector with the intention to create a stable cell line; however, the cells became resistant to neomycin and we were unable to select for individual clones. We decided to seek out a new vector with a selectable marker other than neomycin and found pQCXIP gfp, which is a viral vector used for cloning hairpin siRNA constructs (Fig. 8).

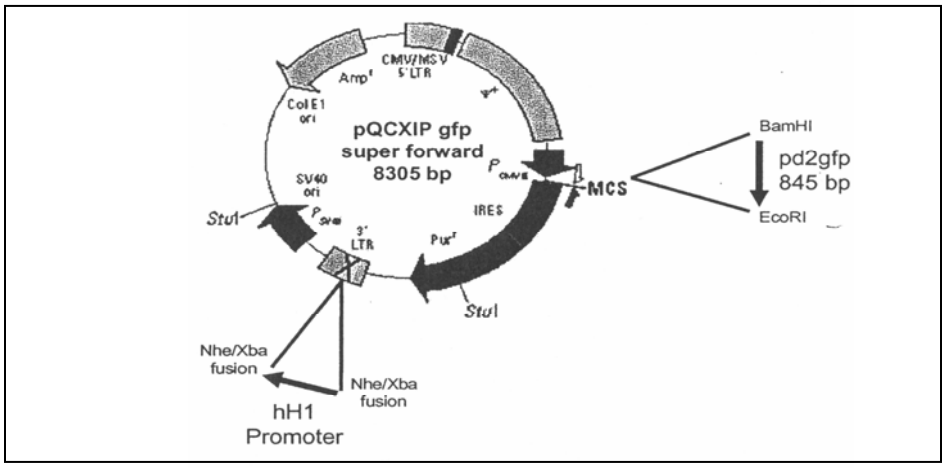

Figure 8. Schematic of pQCXIP gfp viral vector used for cloning siRNA constructs.

In order to test for efficient knockdown with this new vector, we conducted an 11-day selection study in HEK cells and AII protein was assessed on a daily basis. Fig. 9 is a western blot illustrating a decline in AII protein levels over time with the siRNA AII vector compared to vector alone. Now that we have confirmation that we can achieve 100% knockdown with the siRNA AII vector, we are in the process of creating stable cell lines in LNCaP and LAPC-4 cells, since these two prostate cancer cell lines are the most reliant on AII expression.

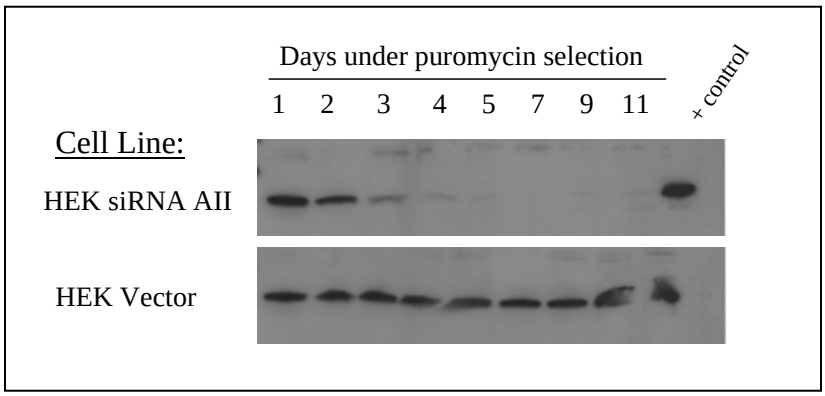

Figure 9. Eleven-day puromycin selection for both HEK siRNA AII and vector.
AII levels assayed using western blot.

Task 3.b Inhibitors Using the Tetracycline-Controlled Transactivator

As stated in our last report, no new work with the tetracycline transactivator is planned at this time. We will be focusing most of our attention on creating stable cell lines expressing the siRNA AII vector mentioned above.

Task 3.c Growth Inhibition and Apoptosis

Our previous attempt to inhibit growth of LNCaP cells with the chemical inhibitor NOHA (*N*-hydroxyarginine) was unsuccessful; however, we speculated that possibly these cells were taking up polyamines from the media which is why we were unable to witness a decline in cell growth rate. To overcome this problem, we obtained a polyamine transport inhibitor compound, ORI 1202, to use in conjunction with NOHA. We set up four experimental groups consisting of native LNCAP cells, LNCaP cells in the presence of 1mM NOHA, LNCaP cells in the presence of 30µM ORI 1202, and LNCaP cells in the presence of 1mM NOHA plus 30µM ORI 1202. Cell counts were taken over a period of five days and compared among all four groups. As evident in Fig. 10, there appears to be no change in cell proliferation rates among the different groups. This confirms our initial observation that NOHA lacks an inhibitory effect on LNCaP cells even in the presence of a polyamine transport inhibitor. Our most influential growth inhibition studies will come from looking at LNCaP cell growth rates in the presence of the siRNA AII vector.

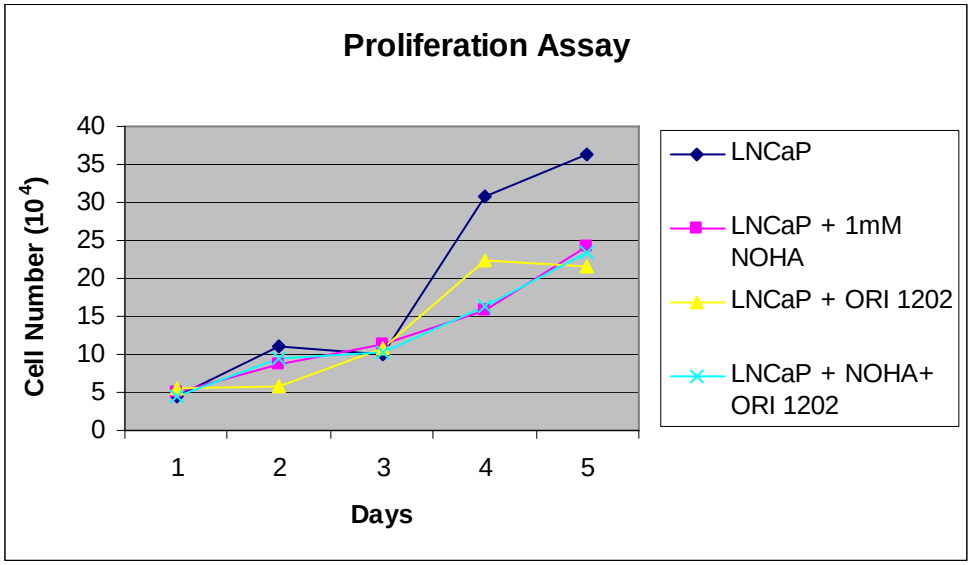

Figure 10. Proliferation assay comparing cell counts between native LNCaP, LNCaP + 1mM NOHA, LNCaP + ORI 1202, and LNCaP + NOHA + ORI 1202.

Task 4.a Arginase Overexpression

We have encountered some difficulty with creating a PZ-HPV-7 stable cell line overexpressing AII. The transfection efficiency appears to be very low for this cell line, which has led us to try several different approaches. Our most current method involves infecting PZ-HPV-7 cells with virus made from a retroviral vector containing the human AII gene. We anticipate the completion of this cell line in the next several months and will begin proliferation and invasion assays comparing the native PZ-HPV-7 cells to PZ-HPV-7 cells overexpressing AII. As stated in previous reports, we have already created and validated several arginase overexpressing prostate cancer cell lines. We did not observe dramatic differences in

proliferation rates between native and arginase overexpressing cell lines, which is most likely due to the rapid basal rate of proliferation of native LNCaP cells and already elevated levels of endogenous AII. Therefore, we feel that the PZ-HPV-7 studies will produce more significant results since the growth rate of these cells is much slower and the endogenous level of AII is considerably lower than in LNCaP cells.

Task 5.a Human Serum

As stated in our last report, our accumulation of serum samples from prostate cancer patients has been delayed because our internal source (UCLA Tissue Procurement Core Laboratory) for these samples no longer has access. We are in search of obtaining the serum from outside sources (an NCI-funded, multi-center biomarker project).

Task 5.b Nude Mice

We have overcome the *Helicobacter* infection of the TRAMP transgenic prostate cancer mouse model and are crossbreeding the AII knockout mice into the TRAMP line to see if any alterations in prostate tumor onset or aggressiveness come about due to the lack of AII expression. Recently, the hybrid animals have reached suitable age at which we could begin dissecting prostates from 23-week-old TRAMP mice and recording their weights. We have observed some heterogeneity in tumor development with these mice so we plan to remove the next set of prostates from TRAMP mice at 30 weeks of age in hopes of eliminating the variation. We will compare prostate weights from native TRAMP mice with those removed from TRAMP/AII knockout mice at 23 and 30 weeks of age. From our crossbreeding efforts, we have generated several TRAMP/AII knockout male mice that will be old enough for these studies in a couple of months. The unavoidably long time periods required for these *in vivo* studies are the reason we have requested a no-cost extension of our USAMRMC support in order to see these experiments through to completion in Year 4.

The proposed nude mouse studies are dependent upon the development of the stable LNCaP cell lines expressing the AII siRNA. Once these cell lines are generated, we will begin injecting LNCaP cells transfected with the AII siRNA into the flank of nude mice and compare these experimental tumors with those formed from untransfected control cells. We are aware of the inconsistent nature of LNCaP cells for growth and metastasis in nude mice (Lee *et al*. 1993), so we will also consider using LAPC-4 or PC3 cells for our xenograft studies.

Key Research Accomplishments

- Expanded our prostate cancer cell work to include two new cell lines and assessed the levels of arginase and several related enzymes through quantitative real-time PCR and western blot analysis.

- Correlated arginase II expression levels with tumor grade looking at individual paraffin sections and tissue microarrays.

- Optimized the laser-capture microscope technique to ultimately uncover the interaction among a multitude of related genes in an individual cell.

- Compared arginase II expression with polyamine levels in the prostate cell lines.

- Constructed a siRNA vector expressing AII that can be used in creating stable cell lines for inhibitory growth studies.

- Performed growth inhibition studies using a chemical inhibitor against arginase in combination with a polyamine transport inhibitor.

- Assessed initial prostate weights in native TRAMP and AII KO mice in preparation for the TRAMP/AII KO crossbred mice to come of age.

- Observed gradient in arginase II expression among microscopic versus macroscopic prostate tumors in the TRAMP mice.

Reportable Outcomes

Publications

Orkin, M.B., Mumenthaler, S.M., Cederbaum, S. and Grody, W. Arginase as a proliferative determinant in prostate cancer. Presented at the Annual Meeting of the Western Society for Clinical Investigation, Carmel, February, 2006. J Invest. Med. **54**: S155, 2006.

Presentations

American Association for Cancer Research: Frontiers in Cancer Prevention Research
Baltimore, Maryland Nov 2005

- Expression of Arginase in Prostatic Adenocarcinoma

We are in the process of writing a manuscript that will be submitted to the Journal of Histochemistry and Cytochemistry. In addition, we are preparing our work for the Annual American Association for Cancer Research conference next year.

Conclusions

During Year 3, our work focused on expanding the previous cell culture and tissue experiments to include new prostate cancer cell lines, a wider array of normal and malignant prostate tissues, and an overall more quantitative analysis. This expansion led us to uncover a more complex interrelationship among arginase and its related enzymes, which we hope to understand in detail at the completion of our laser-capture microscope experiment. We have also extended our comparison of arginase II expression and polyamine levels to include the prostate cell lines in our possession. We have begun work on our *in vivo* mouse models and will continue to gather data as the mice become old enough to study. The proposed *in vitro* and *in vivo* inhibitory studies are underway and the results look promising.

Despite the high incidence of prostate cancer, relatively little is known about the biochemical and molecular mechanisms controlling benign and malignant prostatic growth. This project sets out a novel and original program that seeks to elucidate fundamental underlying mechanisms linking our surprising observation of elevated prostatic arginase AII levels with promotion and potential inhibition of cancer of the prostate. We are building upon our group's long track record of arginase research in the context of a metabolic disorder, arginase deficiency, and are now applying these resources for the first time to an investigation of what we believe to be this enzyme's fundamental involvement in prostate cancer cell growth. This hypothesis is based on the locus of arginase activity at the convergence of critical urea cycle, polyamine synthetic, and nitric oxide pathways, all of which are key aspects of prostate physiology and cell proliferation. We believe this work will enhance our understanding of the fundamental mechanisms of prostatic neoplasia and also suggest new and specific molecular targets for both diagnosis and therapy.

References

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